Suppression among alleles encoding NB-LRR resistance proteins interferes with resistance in F1 hybrid and allele-pyramided wheat plants

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Abstract: Developing high yielding varieties with broad-spectrum and durable disease resistance is the ultimate goal of crop breeding. In plants, immune receptors of the NB-LRR class mediate race-specific resistance against pathogen attack. This type of resistance is often rapidly overcome by newly adapted pathogen races when employed in agriculture. The stacking of different resistance genes or alleles in F1 hybrids or in pyramided lines is a promising strategy to achieve more durable resistance. Here, we identify a molecular mechanism which can negatively interfere with the allele-pyramiding approach. We show that pairwise combinations of different alleles of the powdery-mildew-resistance gene Pm3 in F1 hybrids and stacked transgenic wheat lines can result in suppression of Pm3-based resistance. This effect is independent of the genetic background and solely dependent on the Pm3 alleles. Suppression occurs at the post-translational level as neither RNA nor protein levels of the suppressed alleles are affected. Using a transient-expression system in Nicotiana benthamiana, the LRR domain was identified as the suppression-conferring domain. The results of this study suggest that the expression of closely related NB-LRR resistance genes or alleles in the same genotype can lead to dominant-negative interactions. These findings provide a molecular explanation for the frequently observed ineffectiveness of resistance genes introduced from the secondary gene pool into polyploid crop species and mark an important step to overcome this limitation.

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Title Page

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SUMMARY

Developing high yielding varieties with broad-spectrum and durable disease resistance is the ultimate goal of crop breeding. In plants, immune receptors of the NB-LRR class mediate race-specific resistance against pathogen attack. This type of resistance is often rapidly overcome by newly adapted pathogen races when employed in agriculture. The stacking of different resistance genes or alleles in F1 hybrids or in pyramided lines is a promising strategy to achieve more durable resistance. Here, we identify a molecular mechanism which can negatively interfere with the allele-pyramiding approach. We show that pairwise combinations of different alleles of the powdery-mildew-resistance gene *Pm3* in F1 hybrids and stacked transgenic wheat lines can result in suppression of *Pm3*-based resistance. This effect is independent of the genetic background and solely dependent on the *Pm3* alleles. Suppression occurs at the post-translational level as neither RNA nor protein levels of the suppressed alleles are affected. Using a transient-expression system in *Nicotiana benthamiana*, the LRR domain was identified as the suppression-conferring domain. The results of this study suggest that the expression of closely related NB-LRR resistance genes or alleles in the same genotype can lead to dominant-negative interactions. These findings provide a molecular explanation for the frequently observed ineffectiveness of resistance genes introduced from the secondary

gene pool into polyploid crop species and mark an important step to overcome this limitation.

INTRODUCTION

To prevent yield losses in crop production due to pathogen infestation, breeding for resistant plant genotypes is widely considered to be the most sustainable strategy. Plant breeders constantly renew the set of cultivars offered to the farmers by incorporating resistance loci from the primary, secondary, and tertiary gene pool into breeding germplasm. Molecular cloning of the underlying genetic constituents for pathogen defense has shown that many of the genes with a major resistance effect encode intracellular resistance (R) proteins with an N-terminal coiled-coil (CC) or TOLL/interleukin-1 receptor (TIR), a central nucleotide-binding (NB), and a C-terminal leucine-rich-repeat (LRR) domain (Marone *et al.*, 2013). These proteins, designated NLR, usually provide a strong resistance that is frequently associated with a hypersensitive response (HR), a form of programmed cell death that prevents the spread especially of biotrophic pathogens. NLR proteins are specifically activated by the direct or indirect recognition of avirulence (Avr) molecules that are delivered from the pathogen into the host cell. These are mainly effector proteins that usually support the virulence of the pathogen. Co-evolution of host and pathogen populations leads to diversification or a fast turnover at the genomic *R* and *Avr* loci, resulting in a large allelic diversity and race-specificity of the *R*-gene mediated Effector-Triggered Immunity (ETI) (Dodds and Rathjen, 2010).

Thus, the use of *R*-gene based resistance in crop plants has the drawback of rapid loss of effectiveness. This is especially true for genetically uniform agricultural ecosystems that create a high selection pressure on pathogen populations. Stacking of multiple highly effective, redundantly acting *R* genes each covering a broad race spectrum is considered a promising strategy for a more sustainable use of race-specific resistance in agriculture (Dangl *et al.*, 2013). Due to the redundancy in recognition the pathogen will have to evolve multiple *Avr* genes simultaneously to gain virulence on such *R*-gene-pyramided plants; an unlikely event and, hence, pyramiding is expected to extend the durability of *R*-gene crop resistance (McDonald and Linde, 2002).

As an alternative to the stacking of different *R* genes, different allelic variants of the same *R* gene can also be combined. For dominant *R* genes this is possible in a heterozygous form in F1 hybrids. A genetically stable combination of various alleles can be achieved by the use of transgenic approaches, for example via the cross of transgenic lines having different alleles inserted at random sites. By this approach Bieri *et al.* (2004) selected lines expressing both the *Mla1* and *Mla6* powdery-mildew-resistance specificities from the *Mla* locus in barley. Additive resistance was also obtained when L^6 was combined with the L^2 or L^{10} alleles of a flax-rust-resistance gene in one genotype (Chen *et al.*, 2007). These are promising examples of how, next to the genetic diversity at different loci, also the allelic diversity can be exploited for resistance improvement.

A challenge for the combination of different *R* genes or alleles is their potential functional incompatibility with the genetic background or among the stacked genes/alleles themselves. Incompatibility between resistance genes may result in autoimmunity and this hybrid necrosis sets a barrier for hybridization (Bomblies and

Weigel, 2007). In contrast to this immunity-activating effect, the genetic background may also lead to loss of resistance activity. This resistance-suppression phenomenon is frequently observed and results in a significant limitation for resistance breeding especially in polyploid crop species (e.g., Hanusová *et al.*, 1996; Nelson *et al.*, 1997; Knott, 2000; McIntosh *et al.*, 2011; Liu *et al.*, 2013; Chen *et al.*, 2013 and references therein). Although this is a widespread problem, the underlying molecular determinants and mechanism remained elusive.

In this study we tested the allele-pyramiding approach for chromosome 1AS-localized *Pm3*, a coiled-coil NLR-coding gene that mediates race-specific resistance against powdery mildew (*Blumeria graminis* f. sp. *tritici*, *Bgt*) in wheat and of which 17 functional alleles have so far been described (Yahiaoui *et al.*, 2004; Yahiaoui *et al.*, 2006; Srichumpa *et al.*, 2005; Bhullar *et al.*, 2009; Bhullar *et al.*, 2010). We investigated the pairwise combination of five different alleles of *Pm3* in F1 hybrids and stacked transgenic lines. We show that a quantitative suppression among the *Pm3* alleles themselves frequently limits the efficiency of the resistance combination. We demonstrate that the suppression neither takes place at the transcriptional nor the translational level and that the suppression activity is delimited to the LRR domain. Our results adduce molecular evidence that non-activated alleles of NLR resistance genes can block the activity of their resistant counterparts which has major consequences for their use in crop resistance breeding.

RESULTS

Different *Pm3* alleles cannot be stably combined in one genotype by classical genetics. Therefore we wanted to explore a pyramidization of transgenic *Pm3* alleles to improve powdery-mildew resistance. First, additive or non-additive action of different *Pm3* alleles were tested in the F1 progeny of crosses between lines/cultivars (cv.) that carry different alleles of *Pm3*. Wheat cv. 'Kolibri' carrying *Pm3d* or cv. 'Michigan Amber' (M. Amber) carrying *Pm3f* were crossed with the landrace 'Chul' carrying *Pm3b*. The presence of the two different *Pm3* alleles in the F1 plants was confirmed by PCR amplification of allele-specific *Pm3* markers (Tommasini *et al.*, 2006). For leaf segment infection tests of the F1 hybrid plantlets we selected powdery-mildew isolates that differentiate the resistance specificity of the *Pm3* alleles (Figure 1a): Isolates *Bgt* 97011 and *Bgt* 98229 are avirulent on wheat differential lines for *Pm3d* and *Pm3f* (*AvrPm3d*, *AvrPm3f*), but virulent on *Pm3b* differential lines (*avrPm3b*). In contrast, the isolates *Bgt* 07298 and *Bgt* 07201 are avirulent on *Pm3b* (*AvrPm3b*) but virulent on *Pm3d* (*avrPm3d*) or *Pm3f* (*avrPm3f*), respectively. Previous studies have shown that *Pm3a-f* alleles are dominant resistance genes (Briggle, 1966; Zeller *et al.*, 1993). Assuming additive gene action, we expected the F1 plants which carry both *Pm3* alleles in a heterozygous state to be completely resistant to all of the tested *Bgt* isolates. However, we observed low (10-37% infected leaf area for F1 Chul x Kolibri) to high (60-96% infected leaf area for F1 Chul x M. Amber) levels of infection at 7 days post infection with the isolates *Bgt* 97011 and *Bgt* 98229 (both *AvrPm3d/f*) (Figure 1b and c). The F1 hybrids remained fully resistant towards the *Pm3b*-avirulent isolates *Bgt* 07298 or *Bgt* 07201. The parental cultivars of the crosses, 'Kolibri', 'M. Amber', and 'Chul', displayed complete resistance towards all matching avirulent isolates (Figure 1b and c).

In addition, F1 hybrids between the non-*Pm3*-carrying line 'Bobwhite SH9826' and *Pm3f* line 'M. Amber' remained resistant towards the *AvrPm3f* isolate *Bgt* 97011 (Figure 1c). To investigate whether the genetic background contributes to the observed incomplete resistance in F1 hybrids we crossed *Pm3b*- (Chul/8*CC) and *Pm3f*-near isogenic lines (M. Amber/8*CC). Both lines have cv. 'Chancellor' (CC) as the recurrent parent. F1 plants from these crosses were analyzed by infection tests. As for F1 Chul x M. Amber, we observed full resistance of the F1 Chul/8*CC x M. Amber/8*CC plants towards the *Pm3b*-avirulent isolate *Bgt* 07201 (4% infected leaf area), but also high levels of susceptibility with the *Pm3f*-avirulent isolates *Bgt* 97011 and *Bgt* 98229 (77-97% infected leaf area) (Figure 1b). Overall, these results reveal normal gene function of *Pm3b* (Briggle, 1966; Yahiaoui *et al.*, 2004) but show incomplete resistance in the investigated, two *Pm3* allele-containing F1 hybrids for *Pm3d* and *Pm3f* in one or two genetic backgrounds, respectively. These data suggest that *Pm3d*- and *Pm3f*-mediated resistance can be weakened by the quantitatively acting, negative activity of a suppressor present both in 'Chul' and in the 'Chul'-derived chromosomal regions of Chul/8*CC.

To further study if the genetic background might account for the suppressed resistance we used previously developed transgenic $Pm3a_{HA}$, Pm3b, $Pm3c_{HA}$, $Pm3d_{HA}$, and $Pm3f_{HA}$ lines. They all have the susceptible genetic background of the spring wheat line Bobwhite SH 98 26 where no *Pm3* allele is present (Brunner *et al.*, 2011; Brunner *et al.*, 2012). In addition, we generated transgenic lines $Pm3b_{HA}$ and $Pm3b_{myc}$ in the same genetic background expressing the *Pm3b* allele with a C-terminally fused single hemagglutinin (HA) or c-myc (myc) epitope tag, respectively. All the lines exhibited race-specific powdery mildew resistance over multiple generations.

We used the transgenic Pm3 lines that carry a single *Pm3* allele inserted at random sites in the genome to pyramid the *Pm3* alleles in pairs in *Pm3* double-homozygous lines (Pm3x/y) that stably inherit two *Pm3* alleles. One to three independent crosses between *Pm3* lines were made and the F1 progeny was allowed to self-pollinate for three more generations (F4). The segregation of the individual *Pm3* alleles was analyzed in the F3 or F4 generations with allele-specific *Pm3* markers and double-homozygous lines were selected. For the combination of $Pm3b_{myc}$ with $Pm3f_{HA}$ we additionally selected the corresponding sister lines in the F3 generation, i.e. null segregants for $Pm3f_{HA}$ [$Pm3b_{myc}/(\Delta f_{HA})$] or $Pm3b_{myc}$ [$Pm3(\Delta b_{myc})/f_{HA}$] that are homozygous for either $Pm3b_{myc}$ or $Pm3f_{HA}$, respectively.

The powdery-mildew resistance of all double-homozygous lines was examined alongside with their parental lines (and sister lines for $Pm3b_{myc}/f_{HA}$) in infection tests with three *Bgt* isolates that differentiate the parental resistance specificities. All the parental lines and sister lines exhibited the expected resistance specificities and were either completely resistant (<3% average infected leaf area observed) or completely susceptible (>66% average infected leaf area observed) towards the tested *Bgt* isolates (Figure 2a, Figure S1, Figure S2). In total, seven allele combinations were analyzed in which *Pm3b* combinations were redundantly investigated using the untagged, HA-, and myc-tagged *Pm3b*-fusion variants. Remarkably, $Pm3c_{HA}/d_{HA}$ was highly susceptible to *Bgt* 07298 (*avrPm3d* & *AvrPm3c*); $Pm3a_{HA}$/b, $Pm3a_{HA}/b_{myc}$, $Pm3b/f_{HA}$, $Pm3b_{HA}/f_{HA}$, $Pm3b_{myc}/f_{HA}$, and $Pm3c_{HA}/f_{HA}$ showed intermediate resistance or high susceptibility to *Bgt* 97011 and also in some cases lower resistance to *Bgt* 98229 (both *avrPm3b/c* & *AvrPm3a/f*) (Figure 1a, Figure 2, Figure S1). Here, inoculations with the isolate *Bgt* 97011 always led to a higher level of infection compared to the isolate

Bgt 98229. This shows that the degree of susceptibility in the affected lines depends on the *Bgt* isolate used for infection. This indicates that the diverse virulence potential of different *Bgt* isolates quantitatively influences the fungal infestation on suppressed *Pm3* lines. Summarizing these observations, we found incomplete resistance with one or two isolates in infection tests with *Pm3* double-homozygous lines of four allele combinations. In all these cases only the resistance mediated by one of the two combined *Pm3* alleles was compromised. This is consistent with the observations in the F1 hybrids of the crosses of *Pm3* cultivars (Figure 1b). From these results we infer that the incomplete dominance observed with the F1 of some *Pm3*-cultivar crosses was not due to influences of the genetic background. Instead the incomplete resistance in the F1 plants and the double-homozygous lines is based on negative epistatic effects between the *Pm3* alleles themselves. Interestingly, a reduction of $Pm3f_{HA}$-mediated resistance was observed in all three independent combinations of *Pm3b* with *Pm3f*, but the degree of susceptibility varied depending on the parental *Pm3b* line (e.g., Pm3b/f_{HA} 22%, Pm3b_{HA}/f_{HA} 46%, and Pm3b_{myc}/f_{HA} 77% infected leaf area for *Bgt* 97011). Similar observations were made for the two combinations of *Pm3a* with *Pm3b* (Pm3a_{HA}/b, and Pm3a_{HA}/b_{myc}). Collectively, this dependence on the particular transformed construct or transgenic event shows the quantitative nature of suppression.

For the combinations of *Pm3a* with *Pm3c*, *Pm3a* with *Pm3d*, and *Pm3b* with *Pm3d* we did not detect suppression: Separate infections of the respective double-homozygous lines with three *Bgt* isolates resulted in no or very little infestation (<6% average infected leaf area) (Figure 2b, Figure S2). Thus, while we detected incomplete *Pm3d* resistance with the F1 Chul x Kolibri hybrids (Figure 1b) we did not detect suppression in the Pm3b/d_{HA}, and Pm3b_{myc}/d_{HA} double-transgenic lines. We explain this discrepancy

by the quantitative character of suppression where the suppression may be too weak to cause an obvious loss of resistance in these combinations of *Pm3*-overexpressing transgenic lines, but may be sufficient to compromise the resistance of F1 hybrids expressing the *Pm3* alleles under native conditions. However, it is also possible that suppression is only occurring in a subset of *Pm3*-allele combinations and, therefore, does not affect the allele combinations where we observed additive resistance.

To characterize the molecular basis of the suppression effects we selected the $Pm3b_{myc}$/$Pm3f_{HA}$ combination for which we found strong *Pm3f* suppression and for which the different epitope tags enable allele-specific protein analyses. To test whether the *Pm3* activity is affected by transcriptional silencing we performed $Pm3f_{HA}$- and $Pm3b_{myc}$-allele-specific reverse-transcription-quantitative PCRs (RT-qPCR) with the double-homozygous lines $Pm3b_{myc}/f_{HA}$ and their sister lines from all three crosses. The *Pm3* alleles are expressed from a strong maize ubiquitin promoter in all these lines. From six experiments, two independent ones on each of the three crosses, we detected a minimal (2.1-fold) but significant difference of $Pm3f_{HA}$-expression levels in $Pm3b_{myc}/f_{HA}$ lines compared to the $Pm3f_{HA}$-expressing sister lines $Pm3(\Delta b_{myc})/f_{HA}$ in only one case (Figure 3a). The only significant reduction of $Pm3b_{myc}$ expression in $Pm3b_{myc}/f_{HA}$ lines compared to the respective $Pm3b_{myc}/(\Delta f_{HA})$ sister lines was measured in the same experiment and for the same cross (2-fold reduction) (Figure S3). Given that $Pm3f_{HA}$-mediated resistance is suppressed in all $Pm3b_{myc}/f_{HA}$ lines of all three crosses and that $Pm3b_{myc}$-mediated resistance is not affected in the $Pm3b_{myc}/f_{HA}$ line where we detected a reduced expression, there is no correlation between the resistance phenotypes and differences in *Pm3* expression. Therefore, we conclude that suppression of $Pm3f_{HA}$ in the $Pm3b_{myc}/f_{HA}$ lines is not based on transcriptional silencing.

Next, we wanted to test whether a reduced protein abundance might be the cause for the suppression of *Pm3f*$_{HA}$-mediated resistance. With immunoblots using anti-myc or anti-HA antibodies, respectively, we separately analyzed the PM3B$_{myc}$ or PM3F$_{HA}$ proteins in the double-homozygous Pm3b$_{myc}$/f$_{HA}$ lines in comparison to the sister lines. Similar band intensities indicate that similar levels of PM3B$_{myc}$ and PM3F$_{HA}$ protein are produced in the leaves of Pm3b$_{myc}$/f$_{HA}$ lines and the corresponding sister lines (Figure 3b). This suggests that the incomplete PM3F$_{HA}$-mediated resistance phenotype in Pm3b$_{myc}$/f$_{HA}$ is not correlated with a reduced amount of the PM3F$_{HA}$ resistance protein and indicates a suppression mechanism at the post-translational level.

Some coiled-coil NLR resistance proteins are known to form multimeric complexes already before pathogen perception (Ade *et al.*, 2007; Maekawa *et al.*, 2011). Based on these findings, we presumed that protein interactions might be important for the suppression mechanism and for this reason we analyzed whether protein complexes containing different PM3 proteins can be found in plant cells. We performed co-immunoprecipitation experiments with *Pm3b*$_{myc}$- and *Pm3f*$_{HA}$-co-infiltrated leaf material from *Nicotiana benthamiana* where we previously showed that PM3 is functional (Stirnweis *et al.*, 2014). Here, myc-tagged PM3B$_{myc}$ co-precipitated with HA-tagged PM3F$_{HA}$ protein demonstrating that these two proteins interact (Figure 4a). No similar interaction was detected with the same analysis using primary leaves of the stable transgenic Pm3b$_{myc}$/f$_{HA}$ wheat lines. There, PM3-protein levels are very low and the immunoprecipitation and detection efficiency for PM3F$_{HA}$ or PM3B$_{myc}$, each fused with only a single epitope, may be insufficient. Using *N. benthamiana*, we also tested for the interaction of PM3F$_{HA}$ with myc-tagged hPM3-1B$_{myc}$. This protein is encoded by a homolog of *Pm3* originating from wheat homoeologous chromosome 1B and has 78%

similarity to the PM3B amino-acid sequence (Hurni *et al.*, 2013). The *hPm3-1B* gene is present in cv. 'Chancellor' that was used as recurrent parent in many near-isogenic *Pm3* differential lines and is, therefore, expected to not suppress *Pm3*-mediated resistance. The detection of hPM3-1B$_{myc}$ protein in the PM3F$_{HA}$ precipitate showed that PM3F$_{HA}$ and hPM3-1B$_{myc}$ are also present in a common protein complex (Figure 4a). This indicates that protein interaction with a PM3-like protein *per se* is not sufficient for the suppression of PM3F$_{HA}$.

To examine whether the *Pm3* suppression is independent of factors from the powdery-mildew pathogen we established an assay in *Nicotiana* that allows investigating the phenotypic aspects of PM3 interactions in the absence of mildew: We performed overlapping infiltrations with *Agrobacterium tumefaciens* strains transferring either the construct under investigation or *Pm3f_D501V$_{HA}$*, a version of *Pm3f* coding for an autoactive form of the protein due to a mutation in the MHD motif. This aspartate-to-valine substitution renders many resistance proteins, including PM3, autoactive which leads to the induction of a hypersensitive response (HR) after agroinfiltration (Stirnweis *et al.*, 2014). Hence, the programmed cell death that can be observed after agroinfiltration of *Pm3f_D501V$_{HA}$* resembles a resistance response activated by perception of an avirulent powdery-mildew isolate. The PM3F_D501V$_{HA}$-induced cell death was completely suppressed by PM3B$_{myc}$ in the infiltration overlap at 5 days post infiltration (dpi) while the negative control GUS did not reduce the PM3F_D501V$_{HA}$-mediated HR in the overlapping infiltration zone (Figure 4b). This indicates that the suppression of PM3F by PM3B is independent of components from the powdery-mildew fungus. We also found that PM3F_D501V$_{HA}$-mediated HR was not markedly influenced in overlapping infiltrations with *hPm3-1B$_{myc}$* (Figure 4b). This is in

accordance with the observation that *hPm3-1B* does not appear to interfere with *Pm3*-mediated resistance in wheat. An immunoblot analysis of the proteins in leaf material co-infiltrated with *Pm3f_D501V$_{HA}$* and *Pm3b$_{myc}$* or *hPm3-1B$_{myc}$* and harvested at 43 hours post infiltration (hpi) shortly before the onset of HR shows that the PM3F_D501V$_{HA}$ levels did not significantly differ between infiltrations with and without active suppressor gene (Figure S4a). Overall, these results demonstrate that the *Nicotiana* system recapitulates the suppression effects observed in wheat and reveal that intrinsic protein properties make the difference between the suppressing PM3B and the non-suppressing hPM3-1B.

To investigate which part of the PM3B protein causes suppression we co-infiltrated constructs for fragments of PM3B$_{myc}$ and for PM3F_D501V$_{HA}$ in *N. benthamiana* and examined the HR at 5 dpi. *Pm3f_D501V$_{HA}$* co-infiltration with the Pm3b_CC-NBS$_{myc}$ construct comprising amino acids (aa) 1-602 of PM3B led to an HR that was at least as intense as with the GUS negative control indicating that the CC-NBS domains are not responsible for the suppression but rather enhance the PM3F_D501V$_{HA}$-induced HR (Figure 4c). In contrast, the Pm3b_LRR$_{myc}$ (aa 525-1415) construct comprising the complete LRR domain very efficiently suppressed PM3F_D501V$_{HA}$-mediated HR. We split the LRR in an N-terminal (Pm3b_Sp-LRR15$_{myc}$, aa 525-983) and C-terminal part (Pm3b_LRR15-END$_{myc}$, aa 949-1415) and observed that the HR-suppression property is encoded in the N-terminal fragment. When we shortened the N-terminal fragment, co-infiltration with the construct Pm3b_Sp-LRR12$_{myc}$ (aa 525-879) still showed a strong reduction of PM3F_D501V$_{HA}$-induced HR, whereas with shorter fragments HR suppression was only rarely observed (Pm3b_Sp-LRR10$_{myc}$, aa 525-826) or not detected (Pm3b_Sp-LRR8$_{myc}$, aa 525-774) (Figure 4c). This gradual loss of suppression activity

from Pm3b_Sp-LRR15$_{myc}$ to Pm3b_Sp-LRR8$_{myc}$ may have its origin in the ever shorter size of the fragment or may reflect the importance of the PM3B aa 826-983 for the suppression. An observation that supports the latter hypothesis was that the Pm3b_LRR10-END$_{myc}$ construct (aa 826-1415) frequently displayed suppression in contrast to the Pm3b_LRR15-END$_{myc}$ construct. The construct Pm3b_LRR10-15$_{myc}$ (aa 826-983) was still not sufficient to suppress the PM3F_D501V$_{HA}$-induced HR. Immunoblot analysis of the protein levels in co-infiltrated *Nicotiana* leaves shortly before the onset of HR (~30 hpi for Pm3b_CC-NBS$_{myc}$) showed that PM3F_D501V$_{HA}$ abundance is not significantly altered by suppressing and non-suppressing fragments and all PM3B fragments formed stable proteins (Figure S4b). In addition, infiltrations without *Pm3f_D501V$_{HA}$* showed that none of the *Pm3b* constructs induced HR by itself (Figure S5). In summary, we infer from the deletion analysis that the suppression activity of PM3B towards PM3F is situated in the Spacer-LRR domain and here the N-terminal half plays the major role.

DISCUSSION

The pyramiding of resistance genes or its alleles in a single genotype by the generation of F1 hybrids or genetically stable non-segregating lines is a promising concept for the combination of gene specificities and effectiveness, and for an extension of their durability (McDonald and Linde, 2002; Dangl *et al.*, 2013). Combinations of resistance loci leading to additive gene action have been reported in a number of plant species (e.g., Liu *et al.*, 2000; Hu *et al.*, 2012; Zhu *et al.*, 2012) and also the successful stacking of alleles was reported for two NLR resistance genes (Bieri *et al.*, 2004; Chen *et al.*,

2007). However, there are also a number of reports describing observations of weakened or lost resistance when the source of resistance is introgressed into another genetic background. This is well described for resistance breeding in polyploid crop plants where resistance loci derived from lower-ploidy species of the secondary or tertiary gene pool are often suppressed in the polyploid species or in synthetic polyploids (e.g., Hanusová *et al.*, 1996; Nelson *et al.*, 1997; Knott, 2000; McIntosh *et al.*, 2011; Liu *et al.*, 2013; Chen *et al.*, 2013 and references therein). Incomplete resistance in F1 hybrids, as seen in this study for the *Pm3* F1 hybrids, is also known (e.g., Islam *et al.*, 1992; Wilson and McMullen, 1997; Kim *et al.*, 2012) but the causes were often attributed to gene-dosage dependency. Only few studies so far gave hints that the suppression of a resistance locus might originate from its combination with the corresponding, dominantly acting, susceptible allelic locus: For example, it was shown in *Arabidopsis thaliana* that the TIR-NLR-WRKY-resistance gene *Rrs1*, originally classified as recessive by classical genetics, behaved as a dominant gene when introduced as a transgene (Deslandes *et al.*, 2002). A rust resistance gene of soybean showed dominance in some, but recessiveness in other crosses. There, it was found that the genetic determinant of the suppression co-segregated with the allelic, susceptible resistance locus (Garcia *et al.*, 2011). The results of our study now demonstrate at the molecular level that incompatibility among alleles of an NLR resistance gene can cause resistance suppression.

Combining these findings with those of a companion publication (Hurni *et al.*, 2014; see accompanying manuscript), showing that rye-derived *Pm8*-mediated resistance in wheat can be suppressed by its ortholog *Pm3*, also suggests that other NLR resistance activities might be compromised by closely related NLR proteins (e.g., encoded by

alleles, orthologs, homoeologs, or paralogs) by the same suppression mechanism. Indeed, there are several indications that the identified mechanism how a non-functional resistance protein suppresses a resistant counterpart is of wider significance.

For instance, in hexaploid wheat Nelson *et al.* (1997) genetically mapped the suppressor of chromosome 2B-localized leaf-rust resistance gene *Lr23* to the homoeologous locus on chromosome 2D suggesting a susceptible homeolog of *Lr23* as suppressor. Furthermore, it was shown that expression of a version of the bacterial-NLR-resistance protein RPS2 that is inactivated by mutations in the CC domain has a dominant-negative effect on the wild type RPS2-mediated resistance in *Arabidopsis thaliana* (Tao *et al.*, 2000). Moreover, the viral TIR-NLR-resistance protein N of *Nicotiana* is suppressed by co-expression of N variants inactivated by P-loop mutations, or by deletion of, or mutations in, the TIR domain (Dinesh-Kumar *et al.*, 2000). Similar to our observation for PM3, it was also reported that cell death induced by an autoactive version of the NLR Prf can be suppressed by co-expression of its LRR in a *Nicotiana* infiltration system (Du *et al.*, 2012). Finally, the described suppression mechanism might also be relevant for resistance proteins with an extracellular LRR (eLRR) domain. This hypothesis is based on results of Barker *et al.* (2006) showing that inactive Cf-9 variants with C-terminal deletions in the eLRR have a dominant-negative effect on the wild-type Cf-9 activity in tomato. These examples are all consistent with our findings from the deletion analysis showing that the N-terminal part of the LRR domain is the major determinant of suppression.

Recently, Williams *et al.* (2014) showed for the RPS4/RRS1-NLR pair that heteromeric, TIR-domain independent complexes between these NLR proteins are formed and that co-expression of the RRS1-TIR domain is sufficient to suppress the HR

induced by expression and homodimerization of the RPS4-TIR domain. Our results and data from Du *et al.* (2012) both show that co-expression of the LRR domain is sufficient to suppress the HR induced by an autoactivated full-length NLR protein. These data suggest that multiple domains are potentially involved in the co-suppression and co-activation of NLR-protein complexes (Williams *et al.*, 2014).

The results of this study present molecular evidence that *Pm3* suppression is based on dominant-negative, post-translational effects among the involved proteins. These effects are of quantitative nature as indicated by suppression differences between different *Pm3b* constructs. They are most likely independent of fungal components as suggested by the HR-suppression in the *Nicotiana* infiltration system, and could involve PM3-protein interactions. Thus, we propose the following model for the suppression by alleles: PM3 proteins form complexes, exclusively PM3-homomeric ones when only one allele is present and PM3-homomeric as well as PM3-heteromeric ones when multiple alleles are present. In contrast to homomeric complexes, heteromeric complexes might be incompatible for signaling, or even block it, thereby sequestering the active protein pool of each combined PM3 variant. This also provides an explanation of the quantitative nature of suppression where the ultimate phenotypic outcome depends on the virulence potential of the pathogen race, the individual PM3 protein level and the recognition and activation efficiency of the PM3 proteins encoded by different alleles (Stirnweis *et al.*, 2014). This scenario also implies that the successful pyramiding of the *Pm3a* and *Pm3c*, *Pm3a* and *Pm3d*, and *Pm3b* and *Pm3d* alleles is based on limited quantitative suppression in case of an optimal combination of PM3-protein levels. The observation that hPM3-1B does not suppress PM3, even though the proteins interact as shown by co-immunoprecipitation, indicates that the

inactivation of PM3-protein complexes is a complex process that possibly also depends on particular protein features which differ between PM3B and hPM3-1B.

Overall, the suppression mechanism found in this study is possibly widespread, especially in polyploid species where resistance genes might not only be suppressed by other alleles but also by the homoeologous genes located on the different subgenomes. It can be a limiting factor for gene- or allele-pyramiding approaches as well as for the transfer of resistance genes into species where an ortholog is present. The results of this study suggest that in such cases the mutagenesis, silencing, gene editing, or replacement of the orthologous suppressor gene is a possibility to bypass the unwanted resistance suppression. The introduced *N. benthamiana* infiltration system offers an opportunity to easily verify suppression activities between cloned genes.

EXPERIMENTAL PROCEDURES

Transgenic *Pm3* lines

Five transgenic lines were previously described by Brunner et al. (Brunner *et al.*, 2011; Brunner *et al.*, 2012) and were renamed in this study: $Pm3a_{HA}$ corresponds to Pm3a#1, Pm3b to Pm3b#1, $Pm3c_{HA}$ to Pm3c#1, $Pm3d_{HA}$ to Pm3d#1, and $Pm3f_{HA}$ to Pm3f#1. The cloning and transformation procedures for the $Pm3b_{myc}$ and $Pm3b_{HA}$ transgenic lines are described in Methods S1.

Selection of double-homozygous and sister lines

For the generation of *Pm3* double-homozygous lines up to three crosses between individual plants from two different *Pm3* transgenic lines were made. The resulting F1 progeny was allowed to self-pollinate for three more generations (F4). The segregation of the individual *Pm3* alleles was analyzed in the F3 or F4 generations with allele-specific *Pm3* markers and double-homozygous lines were selected based on segregation analysis using at least 20 individual plants per family of each line. The $Pm3b_{myc}/f_{HA}$ lines as well as the sister lines $Pm3b_{myc}/(\Delta f_{HA})$ and $Pm3(\Delta b_{myc})/f_{HA}$, null segregants for $Pm3f_{HA}$ or $Pm3b_{myc}$ that are homozygous for either $Pm3b_{myc}$ or $Pm3f_{HA}$, respectively, were selected based on marker analysis with at least 30 individuals of one family in the F3 generation.

***Pm3* marker**

Conditions and primers for allele-specific *Pm3* markers were used as described (Tommasini *et al.*, 2006). For the detection of the *Pm3b* and *Pm3d* alleles in the transgenic context the marker primers had to be modified: For *Pm3b* the primer sbi144 (5`-TTTAGCCCTGCCTTCATACG-3`) was combined with the primer Pm3b/R (Tommasini *et al.*, 2006); for *Pm3d* the primers dst003 (5`-AGATGGCAAGCAAGAGGTGT-3`) and dst004 (5`-CAAGCTTAATGCACCCACGA-3`) were used.

Infection tests

Powdery mildew infection tests using leaf segments were performed as previously described by Brunner et al. (2011). Box and Whisker Plots of the obtained data in Figures S1-S2 were created with the software package R (R Core Team, 2013).

RT-qPCR analysis for detection of *Pm3f*$_{HA}$ and *Pm3b*$_{myc}$ expression

Expression of *Pm3f*$_{HA}$ and *Pm3b*$_{myc}$ was separately quantified using a reverse transcription, quantitative real-time polymerase chain reaction (RT-qPCR) assay. Per line, technical triplicates of three biological replicates each were analyzed using a CFX96 Real-Time System C1000^{TM} Thermal cycler (Bio-Rad, www.bio-rad.com). Each biological replicate consisted of three pooled first leaves of 10-day-old plants. *GAPDH* (UniGene Ta.5104) was included as reference gene. For a more detailed description see Methods S1.

Protein extraction and immunoblot analysis

Protein from primary leaves of wheat was detected as essentially described by Brunner et al. (2012) but using the Chemidoc XRS system (Bio-Rad) for blot development instead of x-ray film. Protein detections from *Nicotiana benthamiana* leaves at the indicated time post infiltration were performed as described by Stirnweis et al. (2014). Anti c-myc antibodies (rat monoclonal, clone JAC6, sc-56633; Santa Cruz Biotechnology, www.scbt.com) were used in 1 : 4000 dilution for the detection of c-myc tagged proteins.

Construction of plasmid vectors for agroinfiltrations

Complementary DNA (cDNA) was synthesized on total RNA of cv. Chul or M. Amber/8*CC with the SuperScript III RT (Life Technologies) enzyme according to the manufacturer`s protocol, *Pm3b* or *Pm3f* were amplified by PCR with primers TJ065 (5`-TTGGCGCGCCGCGGATGGCAGAGCGGGTGGTCA-3`) and TJ066 (5`-CCCCCCGGGCGGCCGCTCAGCTCCGGCAGGCC-3`) and were cloned with the StrataClone Blunt PCR Cloning Kit (Agilent Technologies, www.genomics.agilent.com). From these and from existing plasmids all genes were cloned into Gateway system compatible entry vectors via Gateway BP Clonase II reactions (Life Technologies). Introduction of modifications and cloning of fragments were achieved by the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies). By Gateway LR reactions (Life Technologies) all resulting pENTR plasmids were recombined to the binary vector pIPKb004 (Himmelbach *et al.*, 2007) carrying the double-enhanced cauliflower mosaic virus 35S promoter. Detailed primer and cloning information is given in Tables S1, S2 and S3.

Agroinfiltrations and co-immunoprecipitation

Transient expressions of vector constructs in *N. benthamiana* leaves via *Agrobacterium tumefaciens* infiltrations and co-immunoprecipitation experiments were performed according to the protocols of Stirnweis et al. (2014). For overlapping infiltrations *A. tumefaciens* clones containing the $Pm3b_{myc}$, $hPm3\text{-}1B_{myc}$, or *GUS* constructs were infiltrated first and 1-2 h later the infiltrations of $Pm3f\_D501V_{HA}$ were done.

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SHORT LEGENDS FOR SUPPORTING INFORMATION

Figure S1. Results of infection tests with *Pm3* double-homozygous lines showing non-additive gene action.

Figure S2. Results of infection tests with *Pm3* double-homozygous lines showing additive gene action.

Figure S3. *Pm3b$_{myc}$* expression analyses in double-homozygous Pm3b$_{myc}$/f$_{HA}$ lines and the Pm3b$_{myc}$/(Δf$_{HA}$) sister lines.

Figure S4. Immunoblot analyses of PM3 and PM3 fragments after co-infiltrations in *N. benthamiana*.

Figure S5. Pictures of control infiltrations showing that *Pm3b$_{myc}$* fragments and *hPm3-1B$_{myc}$* do not induce cell death induction in *N. benthamiana* infiltrations.

Table S1. Primers used for RT-qPCR.

Table S2. Primers used for the construction of plasmid vectors for agroinfiltrations.

Table S3. Detailed information on the cloning of the Gateway pENTR plasmids.

Methods S1. Supporting information experimental procedures and references

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FIGURE LEGENDS

Figure 1. Suppression among *Pm3* alleles leads to incomplete resistance in F1 hybrids. Hybrids originating from parental lines expressing the *Pm3b*, *Pm3d*, or *Pm3f* allele are incompletely resistant to powdery mildew isolates that are avirulent on the *Pm3d* or *Pm3f* parental lines.

(a) Virulence profiles of *Bgt* isolates used in this study.

(b) Results of leaf segment infection tests with parental lines and F1 hybrids originating from crosses between cultivars expressing *Pm3b*, *Pm3d*, and *Pm3f*. Three independent crosses per hybrid combination (1-3) were analyzed. Infections were done with *Bgt* isolates that differentiate the parental resistance specificities. Data for at least eight replicates is presented with Box and Whisker Plots and the median values are shown with black horizontal lines.

(c) Pictures of leaf segment infection tests taken at 7 days post infection showing suppression of *Pm3f* in F1 Chul x M. Amber. *Bgt* isolates 97011 and 07201 differentiate the *Pm3b*- and *Pm3f*-mediated resistance as shown by the resistance specificity of wheat differential lines Chul and M. Amber. Infections of cultivar Bobwhite SH 9826 (BWS) and F1 BWS x M. Amber are shown as controls.

Figure 2. *Pm3*-mediated resistance is suppressed in several *Pm3*-double-homozygous lines and the level of fungal infestation on the suppressed plants depends on the *Bgt* isolates.

(a) Leaf segment infection tests with *Bgt* isolates 97011, 98229, and 07201 are shown. These isolates differentiate the $Pm3b_{myc}$- and $Pm3f_{HA}$-mediated resistance as shown by the resistance specificity of the $Pm3b_{myc}/(\Delta f_{HA})$ and $Pm3(\Delta b_{myc})/f_{HA}$ sister lines. Pictures were taken at 7 days post infection.

(b) Summary of results of leaf segment infection tests with transgenic lines homozygous for two different *Pm3* alleles and differentiating *Bgt* isolates. Colors of the arrows indicate mean infected leaf areas of the double-homozygous lines for the tested *Bgt* isolates and the corresponding suppression levels of the double-homozygous line

compared to the respective resistant parental line (light gray boxes). The direction of the arrows indicates the suppression activity of the *Pm3* allele on the base of the arrow on the resistance activity originating from the *Pm3* allele on the tip of the arrow. Detailed results of infection tests are presented in Figure S1 and Figure S2.

Figure 3. Suppression of *Pm3f*$_{HA}$ activity in double-homozygous Pm3b$_{myc}$/f$_{HA}$ lines does neither occur at the transcriptional nor at the translational level.

(a) RT-qPCR results for the incomplete resistant Pm3b$_{myc}$/f$_{HA}$ lines and the resistant Pm3(Δb$_{myc}$)/f$_{HA}$ sister lines show only minimal differences of *Pm3f*$_{HA}$-transcript accumulation. Mean values (represented by bars) and single data points (x) of two independent experiments each with three replicates are shown. Values are normalized to the lowest value (set to 1) within each experiment. Significant differences are indicated by asterisks (Student`s *t*-test $P<0.05$; n. s. = non significant)

(b) Immunoblot (IB) analysis of PM3B$_{myc}$ (upper panel) and PM3F$_{HA}$ protein (lower panel) in leaves of Pm3b$_{myc}$/f$_{HA}$ lines and the respective sister lines from three independent crosses. Ponceau S membrane staining of Ribulose-1,5-bis-phosphate carboxylase/oxygenase is shown as control for equal loading of total protein.

Figure 4. Agroinfiltration experiments in *Nicotiana benthamiana* reveal that PM3B$_{myc}$ and its homolog hPM3-1B$_{myc}$ physically interact with PM3F$_{HA}$, but only PM3B$_{myc}$ suppresses PM3F$_{HA}$-mediated HR via its LRR domain.

(a) Co-immunoprecipitation of c-myc-tagged PM3B or hPM3-1B with HA-tagged PM3F from co-infiltrated *N. benthamiana* leaves. Images of immunoblots (IB) before (two upper panels) and after (two lower panels) immunoprecipitation (IP) with anti-HA agarose beads are shown.

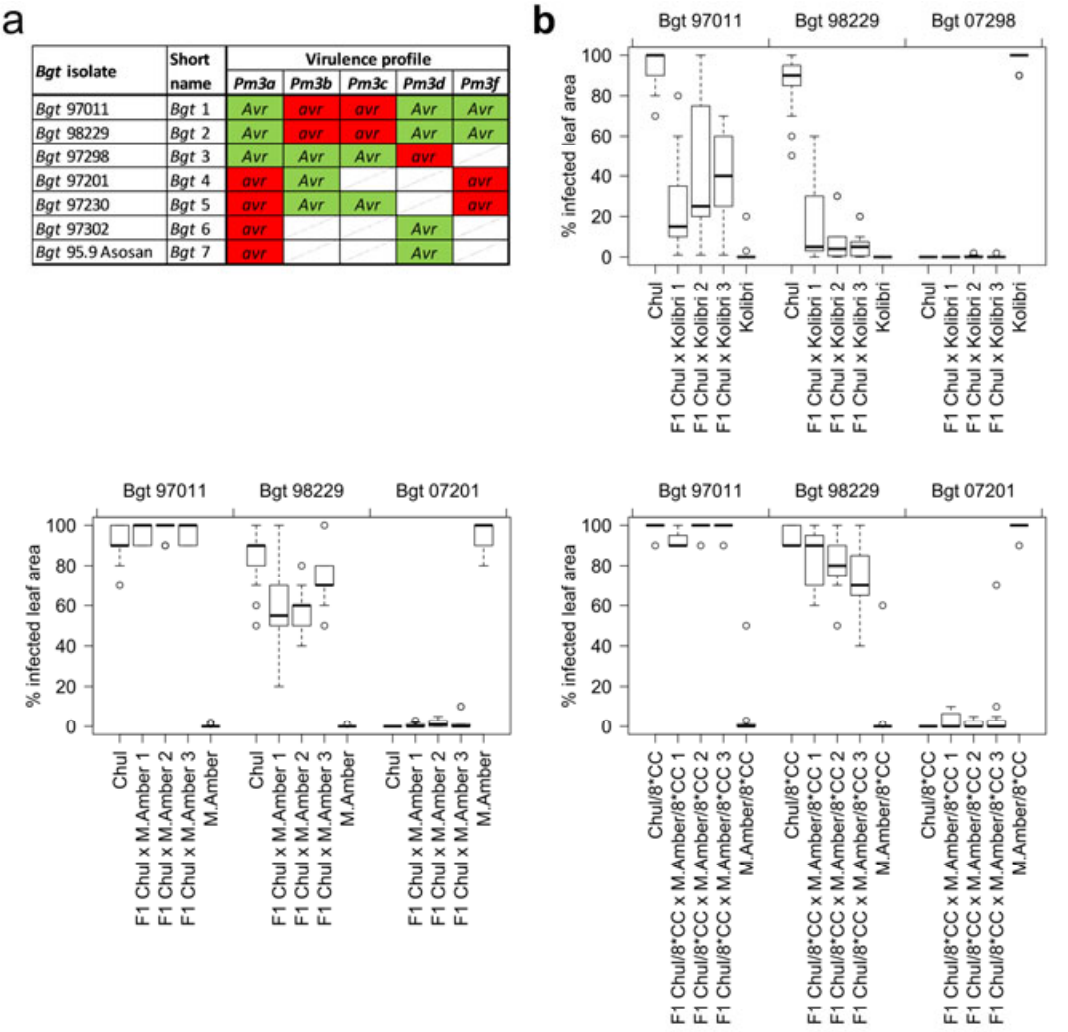
(b) Overlapping infiltrations of autoactive, HR-inducing *Pm3f_D501V$_{HA}$* (upper circles) with *Pm3b$_{myc}$*, the negative control *GUS*, and *hPM3-1B$_{myc}$* (lower circles) for the detection of phenotypic interactions in the overlap. Pictures were taken at 5 dpi.

(c) Deletion analysis for PM3B$_{myc}$ suppression activity. Constructs for PM3B$_{myc}$ fragments and Pm3f_D501V$_{HA}$ were co-infiltrated and HR suppression was scored in comparison to a co-infiltration with the negative control (GUS) at 5 dpi in at least three independent experiments with at least four replicates per experiment (0 = no HR suppression → ++++ = consistently complete HR suppression). Exemplary pictures are shown. Due to variable phenotypes these pictures cannot be representative for all co-infiltrations. The top row of the drawing shows the PM3 domain structure with the CC (blue), NB (red), Spacer (Sp, gray) and LRR (green) domains. Black bars indicate the portion of PM3B encoded by the respective construct. Numbers give the respective positions in PM3B of the outermost amino acids of the fragments.

a

Bgt isolate	Short name	Virulence profile				
		Pm3a	*Pm3b*	*Pm3c*	*Pm3d*	*Pm3f*
Bgt 97011	*Bgt* 1	*Avr*	*avr*	*avr*	*Avr*	*Avr*
Bgt 98229	*Bgt* 2	*Avr*	*avr*	*avr*	*Avr*	*Avr*
Bgt 97298	*Bgt* 3	*Avr*	*Avr*	*Avr*	*avr*	
Bgt 97201	*Bgt* 4	*avr*	*Avr*			*avr*
Bgt 97230	*Bgt* 5	*avr*	*Avr*	*Avr*		*avr*
Bgt 97302	*Bgt* 6	*avr*			*Avr*	
Bgt 95.9 Asosan	*Bgt* 7	*avr*			*Avr*	

b



c